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NBGNU: a hypoxia-activated tripartite combi-nitrosourea prodrug overcoming AGT-mediated chemoresistance

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Aim: A hypoxia-activated combi-nitrosourea prodrug, *N*-(2-chloroethyl)-*N'*-2-(2-(4-nitrobenzylcarbamate)-*O*⁶-benzyl-9-guanine)ethyl-*N*-nitrosourea (NBGNU), was synthesized and evaluated for its hypoxic selectivity and anticancer activity *in vitro*. **Results:** The prodrug was designed as a tripartite molecule consisting of a chloroethylnitrosourea pharmacophore to induce DNA interstrand crosslinks (ICLs) and an *O*⁶-benzylguanine analog moiety masked by a 4-nitrobenzylcarbamate group to induce hypoxia-activated inhibition of *O*⁶-alkylguanine-DNA alkyltransferase. NBGNU was tested for hypoxic selectivity, cytotoxicity and DNA ICLs ability. The reduction product amounts, cell death rates and DNA ICL levels induced by NBGNU under hypoxic conditions were all significantly higher than those induced by NBGNU under normoxic conditions. **Conclusion:** The tripartite combi-nitrosourea prodrug exhibits desirable tumor-hypoxia targeting ability and abolished chemoresistance compared with the conventional chloroethylnitrosoureas.

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Chloroethylnitrosoureas (CENUs), a vital type of bifunctional alkylating agent, are efficacious against leukemia and various solid tumors, including brain tumors, melanomas and lymphomas [1–3]. Numerous pharmacological investigations have demonstrated that CENUs exert their anticancer effect by covalently connecting the complementary DNA bases to form interstrand crosslinks (ICLs). As shown in Figure 1, CENUs undergo spontaneous decomposition *in vivo* to release an isocyanate inducing carbamylation of proteins and a chloroethylating cation attacking the *O*⁶-site of guanine. The yielded *O*⁶-chloroethylguanine (*O*⁶-ClEtG) further under cyclization to form a *N*1,*O*⁶-ethanoguanine intermediate, which attacks the complementary cytosine residue on *N*3-site, leading to the formation of dGuo-dCyd crosslinks. DNA ICLs hinder DNA replication and transcription by interrupting the separation of double-stranded DNA and consequently result in tumor cell growth inhibition [4]. However, a significant decrease in the chemotherapeutic effect of CENUs has frequently been observed in their clinical application, which was demonstrated to be due to the suppression of DNA ICLs mediated by *O*⁶-alkylguanine-DNA alkyltransferase (AGT) [5–7]. AGT is a unique enzyme that restores normal guanine by removing the alkyl groups from *O*⁶-site of guanine. The two intermediates induced by CENUs, *O*⁶-ClEtG and *N*1,*O*⁶-ethanoguanine, can be repaired by AGT, thereby blocking the formation of dGuo-dCyd crosslinks. As a result, cells expressing low AGT level exhibit high sensitivity CENUs, whereas cells expressing low AGT level show drug resistance to CENU chemotherapeutics [8]. To improve the cytotoxicity of the alkylating agents modifying *O*⁶-site of guanine, great efforts have been devoted to the development of AGT inhibitors, which were employed as chemotherapeutic adjuvants in clinical trials or in preclinical studies [7,9].

As the first AGT inhibitor reaching clinical trials, *O*⁶-benzylguanine (*O*⁶-BG) effectively dissipates the activity of AGT and sensitizes cancer cells to chemotherapies [10–16]. *O*⁶-(4-Bromothienyl)guanine, a more efficient

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Figure 1. General mechanism for the formation and repair of dGuo-dCyd crosslinks induced by chloroethylnitrosoureas.

AGT inhibitor than O^6 -BG by ten-times higher potency, has also entered clinical trials [17,18]. Among several methyl- and aminomethyl-substituted O^6 -BGs that were also synthesized to inhibit AGT activity, O^6 -[(2-aminomethyl)benzyl]guanines exhibited the highest potency because of an additional H-bond connecting the meta-aminomethyl moiety with active center of AGT [19]. In addition to O^6 -BG derivatives, some pyrimidine compounds exhibit potent AGT inhibition and therefore can reduce AGT-mediated drug resistance. Chae *et al.* [20] observed that 6(4)-(benzyloxy)pyrimidine derivatives possessed more potent AGT-inhibition than O^6 -BG, enabling them to significantly improve the anticancer efficacy of 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea (nimustine, ACNU) [21]. In addition to the application of chemotherapeutic adjuvants, a number of novel chemotherapies with decreased drug resistance were designed based on the concept of 'combi-molecules' by combining two functional groups into one molecule [22–28]. Two combi-nitrosourea prodrugs recently synthesized in our laboratory showed higher potency against SF763 glioma cells and induced more DNA ICLs than traditional CENU chemotherapies owing to the O^6 -BG derivative pharmacophores inhibiting AGT-mediated DNA repair [29]. However, since neither the AGT inhibitors nor the combi-nitrosourea prodrugs mentioned above were designed with the ability to target tumor cells, they were observed or predicted to unavoidably cause toxicity to normal cells and increase myelosuppression toxicity. To overcome this problem, some combi-molecules with one or more pharmacophores selectively acting on tumor tissues were developed as tumor-targeting chemotherapies.

Hypoxia area is ubiquitous in solid tumors caused by insufficient vascularization and increased oxygen consumption due to cancer cell proliferation. Although tumor hypoxia correlates with advanced stages of malignancy, it can be utilized to discover novel hypoxia-targeting chemotherapeutics [30]. Hypoxia-activated prodrugs, which undergo hypoxic activation via a common mechanism involving one- or two-electron reduction, have attracted great interest in

the past three decades. Several hypoxia-activated prodrugs with promising hypoxic selectivity have progressed to pre-clinical and clinical trials, including SN30000, TH-302, EO9, AQ4N, PR-104, tirapazamine and tarloxotinib [31]. Sartorelli's group performed profound investigations on the development of hypoxia-activated O^6 -alkylating agents and AGT inhibitors and provided in-depth insights into the design, structure–activity relationships and mechanisms of action of these prodrugs [32]. They synthesized a series of hypoxia-activated bis(sulfonyl)hydrazine prodrugs as alkylating agents, including KS119, PNBC and KS900. KS119 and PNBC [33–35] exhibited significant preferential toxicity to hypoxic EMT6 and CHO cells, and KS119 could overcome the resistance to O^6 -guanine alkylating agents in oxygen-deficient cells. Designed and synthesized as a hypoxia-activated methylating agent, KS900 [36] sensitized repair-proficient tumor cells to chloroethylating agents by selectively ablating AGT under hypoxic conditions. In addition to hypoxia-activated bis(sulfonyl)hydrazine prodrugs, a series of hypoxia-targeted AGT inhibitors were also reported by Sartorelli's group. They synthesized 4-nitrobenzyloxycarbonyl prodrug derivatives of O^6 -BG and demonstrated their ability to undergo hypoxic activation, which selectively enhanced the sensitivity of DU145 cells with high AGT expression to laromustine under hypoxia but not normoxia [37,38]. Another hypoxia-activated AGT inhibitor, 2-nitro-6-benzyloxypurine, was also demonstrated to deplete AGT activity in intact cells under hypoxia, which selectively sensitized cancer cells to laromustine [39].

In this study, a novel hypoxia-activated combi-nitrosourea prodrug, *N*-(2-chloroethyl)-*N*'-2-(2-(4-nitrobenzylcarbamate)- O^6 -benzyl-9-guanine)ethyl-*N*-nitrosourea (NBGNU), was synthesized and its antitumor activity was evaluated. The compound acts as a tripartite prodrug due to its CENU pharmacophore and O^6 -BG analog moiety masked by a hypoxia-activated 4-nitrobenzylcarbamate group, which released the chloroethylating intermediate to induce dGuo-dCyd crosslinks and the hypoxia-activated O^6 -BG derivative selectively inhibited AGT in the oxygen-deficient region of solid tumors. To evaluate the hypoxic selectivity, we compared the enzymatic reduction, cytotoxicity to human glioma cells and dGuo-dCyd crosslinking levels induced by NBGNU under hypoxic and normoxic conditions. The tripartite combi-nitrosourea is designed to exhibit tumor-targeting ability and is expected to eliminate chemoresistance.

Material & methods

Materials & measurements

All chemicals and enzymes were purchased from J&K Scientific Ltd. (Shanghai, China), TaKaRa Biotech (Tokyo, Japan), Sigma-Aldrich Chemical Co. (WI, USA) and Beijing Chemicals Co. (Beijing, China). The synthesis of NBGNU and deuterated O^6 -benzyl-*N*9-(2-amino)ethylguanine (D_6 -AEBG) was stated in the Supplementary Materials. The standard of 1-[*N*3-deoxycytidyl],2-[*N*1-deoxyguanosinyl]ethane (dGuo-dCyd cross-link) and the isotope-labeled $^{15}N_3$ -dGuo-dCyd internal standard were prepared according to our previous studies [40,41]. All synthesized compounds were confirmed by UV, IR, 1H NMR and high-resolution mass spectrometry. HPLC–ESI–MS/MS quantitation was performed on a triple quadrupole mass spectrometer (TSQ Quantum Discovery MAX, Thermo Finnigan, CA, USA).

Hypoxic activation of NBGNU in phosphate-buffered saline

Hypoxic activation of NBGNU in phosphate-buffered saline (PBS) was examined under enzymatically generated oxygen-deficient conditions according to a method described in previous studies with minor modification [33,42]. Glucose and glucose oxidase were added to the mixture to consume oxygen, catalase was used to get rid of H_2O_2 , and xanthine/xanthine oxidase was employed to provide a reducing environment. The mixture finally consists of 10 mM glucose, 10 units/ml glucose oxidase, 600 units/ml catalase, xanthine and xanthine oxidase at certain concentrations in PBS (100 mM, pH 7.4). NBGNU solution was added into the hypoxic reaction system to give a concentration of 20 μ M. Then, the centrifuge tube containing the mixture was flushed with nitrogen for 10 s to exclude oxygen and then was sealed promptly. The contents of the normoxic samples were identical to those of the hypoxic samples except that glucose addition, which was necessary for the oxygen consumption, was omitted. Air was bubbled into the aerobic reaction mixtures for 10 s, and the tubes were also sealed. After incubation at 37°C for 2, 4, 6 and 8 h, 0.5 of acetonitrile was added to all samples, which were then centrifuged at $10000 \times g$ for 10 min to remove proteins. The supernatant was collected, D_6 -AEBG (40 nM) was added as an internal standard, and the resultant solution was stored at -20°C for HPLC–MS/MS quantitation.

Figure 2. Mechanisms of the induction of DNA interstrand crosslinks under hypoxia condition, chemoresistance inhibition under normoxia condition and hypoxic activation of NBGNU.

Hypoxic activation of NBGNU in cell cultures

Hypoxic activation in cell suspensions was also examined to clarify the influence of the cells and the culture medium on the enzymatic reduction of NBGNU. Before exposure to NBGNU under hypoxia, human brain glioma SF126 cells were pretreated with *O*⁶-BG (50 μM) for 2 h to completely deplete AGT activity. Hypoxic reaction mixtures were prepared as described above. Freshly prepared NBGNU solution was added to the cell suspensions to give a concentration of 0.2 mM. Then, nitrogen was blown into the centrifuge tubes for 30 s to exclude oxygen (purge above and below the surface of the liquid for 20 and 10 s, respectively), followed by promptly sealing the tubes with airtight caps. The normoxic groups were treated identically to the hypoxia groups except that the glucose oxidase was omitted since the DMEM contained 25 mM glucose. Air was blown into the reaction mixtures for 30 s to maintain aeration and then the tubes were sealed. The cell suspensions were incubated at 37°C for 1, 2, 4 and 8 h with gentle shaking, followed by adding an equal volume of acetonitrile and centrifuged at 10,000 × *g* for 10 min. The supernatant was collected, *D*₆-AEBG (40 nM) was added as an internal standard. The obtained solutions were stored at -20°C for HPLC–MS/MS quantitation.

HPLC–MS determination of the reduction product in PBS & in cell cultures

The contents of the reduction product, AEBG (compound **6** in Figure 2), yielded from enzymatic reduction of NBGNU in PBS and in cell suspensions were determined using HPLC–MS/MS. A reverse phase column (ZORBAX SB C18, column size $2.1 \times 150 \text{ mm}^2$; particle size $5 \text{ }\mu\text{m}$; Agilent Technologies, CA, USA) was utilized for HPLC analysis. The mobile phases consisted of 50 mM ammonium acetate (pH 4.5) as solvent A and

acetonitrile as solvent B with a flow rate of 0.1/min. The gradient of the mobile phases was started from 20% B followed by a linearly increase to 35% B within 20 min. Then solvent B was raised up to 90% B in 5 min followed by being held at 90% B for 3 min. Finally, the mobile phase was brought back to the initial composition (20% B) in 2 min. The amounts of the hypoxia generated AEBG were quantified by selecting reaction monitoring (SRM) with the transitions of m/z 285 $\rightarrow$ m/z 242 for AEBG and m/z 291 $\rightarrow$ m/z 248 for D_6 -AEBG.

Cell viability assay

Cell viability assay was determined by the Cell Counting Kit-8 (CCK-8, Dojindo China CO., Ltd, Shanghai, China) assay. Before treatment with drugs, human glioma SF763 cells with high AGT expression (119 fmol/10⁶ cells) [43,44] were seeded as previous described [45]. Then the cells were incubated in fresh medium containing drugs with proposed concentrations for 24 h. The combination-treated groups were pretreated by O^6 -BG (20 μ M) for 2 h before exposure to ACNU for 24 h. Subsequently, cells were treated with 10% CCK-8 reagent in fresh medium for 4 h, followed by measuring the absorbance at 450 nm. The cell viability was calculated according to Equation 1. Here A_{treat} is the absorbance values of the drug-treated cells, A_{blank} is the absorbance values of blank only containing medium and A_{control} refers to the absorbance values of the cells without drug treatment.

$$\text{Cell Viability(\%)} = (A_{\text{treat}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\% \quad (\text{Equation 1})$$

Determination of dGuo-dCyd crosslinks in cells

Stock solutions of ACNU and BGCNU were freshly prepared and were diluted with DMEM to the final concentrations of 0.2 and 0.6 mM. Human glioma cells (SF763 and SF126) were treated with the drugs at 37°C for 12 h under normoxia and hypoxic conditions. For the combination treatment groups, cells were pretreated by O^6 -BG (20 μ M) for 2 h followed by exposure to 0.2 and 0.6 mM ACNU for 12 h. As described above, the hypoxic condition was enzymatically generated using the glucose oxidase/catalase/glucose mixture containing 10 units/ml glucose oxidase to consume oxygen, 600 units/ml catalase to remove the generated hydrogen peroxide and 8 units/l xanthine oxidase plus 50 μ M xanthine to provide reduction condition for BGCNU [33,43]. Nitrogen was utilized to blows away oxygen from the flasks followed by promptly sealing with airtight caps. For the normoxia-treated groups, the reaction mixture was identical to the hypoxia-treated groups except that the glucose oxidase needed for the oxygen consumption was omitted. The negative control groups were incubated without drugs under the same conditions as the treated groups. Then the obtained cellular pellets were used for DNA isolation and enzymatic hydrolysis according previous studies [29,45–48], which was described in the Supplementary Materials.

HPLC–MS analysis of dGuo-dCyd crosslinks

The dGuo-dCyd crosslinks in DNA enzymatic hydrolysis was quantified by HPLC–ESI–MS/MS as previously described with minor modification [29,45,46]. Briefly, the analytes were separated using a reverse phase column (ZORBAX SB C18, column size 2.1 $\times$ 150 mm²; particle size 5 μ m; Agilent Technologies, CA, USA). The mobile phases is composed of deionized water as solvent A and acetonitrile as solvent B. The gradient of the mobile phases was started from 2% B and then increased to 80% B in 25 min. The solvent composition was held at 80% B for 3 min, followed by being brought back to 2% B in the next 2 min. The flow rate of the mobile phase was 0.1/min. The parameters of MS analysis were set as follows: positive mode on an ESI source; 3500 V for the spray voltage; 35 psi for the sheath gas (nitrogen) pressure; 5 psi for the auxiliary gas (nitrogen) pressure; 300°C for the capillary temperature; 107 V for the tube lens offset; 10 V for the source collision induced dissociation; and 20 eV for the collision energy. The levels of dGuo-dCyd crosslink were determined by SRM with the transitions of m/z 521 $\rightarrow$ m/z 289 for dGuo-dCyd and m/z 524 $\rightarrow$ m/z 292 for ¹⁵N₃-dGuo-dCyd. The levels of dGuo-dCyd crosslinks were expressed as fmol dGuo-dCyd crosslinks per mg DNA and all data were processed by statistical analysis described in the Supplementary Materials.

Molecular docking

Molecular docking was used to explore the inhibitory action of AGT under hypoxia and normoxia. The interactions between AGT and the O^6 -BG analogs released from the decomposition and hypoxic activation of NBGNU were simulated using GOLD software. The geometric structures of all ligands were optimized using Gaussian 09 program package (Gaussian, Inc., CT, USA) at the B3LYP/6-31g+(d,p) theoretical level [49]. Human AGT (PDB ID code:

1QNT) x-ray crystal structure was acquired from the Protein Data Bank. Gold Score was used for the selection of the most stable docking models.

Results

Chemistry

In this study, we report a novel hypoxia-activated combi-nitrosourea prodrug, *N*-(2-chloroethyl)-*N'*-2-(2-(4-nitrobenzyl-carbamate)-*O*⁶-benzyl-9-guanine)ethyl-*N*-nitrosourea (NBGNU, compound **1** in Figure 2), which featured three moieties with different functions. As shown in Figure 2, the nitrosourea moiety undergoes decomposition to release a chloroethyl diazonium ion and leads to dGuo-dCyd ICLs similar to traditional CENU chemotherapeutics, the 4-nitrobenzyloxycarbonyl group acts as a hypoxia-activated trigger by masking the 2-amino group of *O*⁶-BG and the *O*⁶-BG analog moiety exerts AGT inhibitory activity by unmasking the 2-amino group under hypoxia to enhance the sensitivity of cells to the nitrosourea pharmacophore. Therefore, the combi-nitrosourea prodrug is designed to selectively sensitize cells to the therapeutic effect of the CENU pharmacophore under hypoxic conditions, which leads to the targeting action toward the hypoxic area in solid tumors.

Stability of NBGNU

Since CENUs undergo spontaneous decomposition in aqueous solution and release diazonium ions or carbon cations as alkylating agents, the degradation of NBGNU was monitored by HPLC–ESI–MS/MS in MEM-EBSS culture medium. NBGNU was monitored by SRM of the transitions *m/z* 598 → 4 *m/z* 90. The retention time of NBGNU was 12.2 min. As shown in Figure 3, the half-life of NBGNU in MEM-EBSS was 18 min, which was slightly longer than the 15 and 12 min half-lives of BCNU (in plasma) and ACNU (in plasma), respectively and was similar to those reported for combi-nitrosoureas (in cell culture media) in our previous studies [29,45,50,51].

Enzymatic reduction of NBGNU under hypoxia

The hypoxic microenvironment in solid tumors was simulated to determine the enzymatic reduction *in vitro* of NBGNU in PBS and in cell suspension using HPLC–ESI–MS/MS. Typical SRM chromatograms of the reduction product AEBG in PBS and in cell suspension are shown in Figure 4A–D. The peak corresponding to AEBG coeluted with the *D*₆-AEBG internal standard at a retention time of approximately 14 min, whereas AEBG was not observed in the normoxic group in PBS (Figure 4A). As shown in Figure 4E, the yield of AEBG was significantly dependent on the concentration of reductase but was slightly influenced by the incubation time after 1 h.

Unlike in PBS, AEBG was observed under both hypoxic and normoxic conditions in cell suspension (see Figure 4C & D), but the levels under normoxia were very low (<0.1 μM). This indicated that NBGNU was effectively reduced in cell suspension under hypoxia, but also slightly reduced under normoxia, which was consistent with the observation of the reduction of nitrobenzene derivatives reported in previous studies [34–36,43,52]. Figure 4F shows the amount of AEBG yielded in cell suspension. Despite the presence of the reduction product under aerobic condition, the production of AEBG under hypoxia was significantly higher (*p* < 0.01) than that under normoxia by 106–134-times, which suggested that NBGNU exhibited obvious hypoxic selectivity in cell suspension.

Sensitivity of human glioma cells to NBGNU

To confirm the hypoxic selectivity of NBGNU, cell viability assays were performed using human glioma SF763 cells (high AGT expression). The cytotoxicity of NBGNU was compared with that of ACNU and ACNU plus *O*⁶-BG under hypoxia and normoxia. As shown in Figure 5A, significantly higher death rates were observed under hypoxia than under normoxia in the NBGNU-treated groups. The IC₅₀ value of NBGNU under hypoxia is 126 μM, which is 4.6-times lower than that under normoxia (580 μM). As expected, there is no obvious difference between the hypoxic and normoxic groups treated with ACNU and with ACNU plus *O*⁶-BG owing to the absence of hypoxic selectivity. In the groups treated with ACNU and ACNU plus *O*⁶-BG, the IC₅₀ values of the treatments were higher than 1000 μM, >8-times higher than that of NBGNU under hypoxia. The results indicated that SF763 cells were effectively sensitized by the AGT inhibitor generated under hypoxic conditions, which resulted in higher cytotoxicity of NBGNU than ACNU and its combination with *O*⁶-BG.

Quantification of dGuo-dCyd crosslinks using HPLC–ESI–MS/MS

In our previous studies, the levels of dGuo-dCyd crosslink were observed to be positively correlated with the death rate of tumor cells treated with CENUs, indicating that the level of dGuo-dCyd crosslinks was correlated with

Figure 3. Decomposition of NBGNU monitored by HPLC-ESI-MS/MS. (A) Typical SRM chromatograms of NBGNU coeluted with the deuterated internal standards. **(B)** Degradation of NBGNU in MEM-EBSS cell culture medium over 8 h of incubation.

the anticancer efficacy of CENUs [46,53]. Therefore, the dGuo-dCyd crosslinks in human glioma cells exposed to NBGNU, ACNU and ACNU plus *O*⁶-BG were determined using HPLC-ESI-MS/MS. Figure 5B & C shows the SRM chromatograms of dGuo-dCyd and ¹⁵N₃-dGuo-dCyd internal standard in cellular DNA enzymatic hydrolysates. The peak of the dGuo-dCyd crosslinks coeluted with ¹⁵N₃-dGuo-dCyd at the retention time of 20 min. In contrast, no signal corresponding to the dGuo-dCyd crosslinks was detected in the control cell samples, which suggested that the internal standard and components of the enzymatic hydrolysates did not interfere with or contaminate the analyte channels.

The levels of dGuo-dCyd crosslink determined in DNA samples from drug-treated SF763 and SF126 cells under normoxia and hypoxia are shown in Figure 5D–G. Prominent levels of dGuo-dCyd crosslink were observed in the samples exposed to NBGNU under hypoxia, and these levels were significantly higher (*p* < 0.01) than the NBGNU-exposed samples under normoxia and those in the groups treated with ACNU or ACNU plus *O*⁶-BG

Figure 4. Reduction of NBGNU under normoxic and hypoxic conditions. Typical SRM chromatograms of AEBG and D₆-AEBG (40 nM) as an internal standard for NBGNU treatments (A) in phosphate-buffered saline (PBS) under normoxia containing 10 units/ml glucose oxidase, 600 units/ml catalase, 5 mM xanthine and 0.8 U/ml xanthine oxidase; (B) in PBS under hypoxia containing 10 mM glucose, 10 units/ml glucose oxidase, 600 units/ml catalase, 5 mM xanthine and 0.8 U/ml xanthine oxidase; (C) in SF763 cell suspensions under normoxia containing 25 mM glucose, 600 units/ml catalase; and (D) in SF763 cell suspensions under hypoxia containing 25 mM glucose, 10 units/ml glucose oxidase, 600 units/ml catalase. Production of AEBG from the reduction of NBGNU under hypoxic conditions induced (E) by glucose (10 mM)/glucose oxidase (10 units/ml)/catalase (600 units/ml) and xanthine/xanthine oxidase in PBS and (F) by glucose oxidase (10 units/ml)/catalase (600 units/ml) without xanthine/xanthine oxidase in cell suspension. The concentration of NBGNU is 20 μ M and the exposure times for (A–D) are all 2 h. An amount of 40 nM deuterated AEBG was added. SRM: Selecting reaction monitoring.

under both conditions. For SF763 cells (Figure 5D & E), the levels of NBGNU-induced dGuo-dCyd crosslink under hypoxia were 3.7 and 4.2-times higher ($p < 0.01$) than those under normoxia for the low (0.2 mM) and the high (0.6 mM) dose groups, respectively. The levels of dGuo-dCyd crosslink induced by NBGNU under hypoxia were 17.2–40.2 and 6.0–9.8-times higher ($p < 0.01$) than those induced by ACNU and ACNU plus *O*⁶-BG, respectively. In contrast to the groups treated with NBGNU, we did not observed significant difference between the normoxic and the hypoxic groups treated with ACNU or with ACNU plus *O*⁶-BG. For SF126 cells (Figure 5F & G), the levels of NBGNU-induced dGuo-dCyd crosslink under hypoxia were 1.9-times higher ($p < 0.01$) than those under normoxia for both low and high dose groups, although the difference between hypoxic and normoxic conditions was not as remarkable as that for the SF763 cells. Moreover, the levels of dGuo-dCyd crosslink induced by NBGNU under hypoxia were significantly higher ($p < 0.05$) than those of the ACNU and ACNU plus *O*⁶-BG groups. The results demonstrated that under hypoxic conditions, AGT activity was dramatically decreased by the

Figure 5. Cell viability and DNA ICLs in human glioma cells treated with NBGNU under normoxia and hypoxia. (A) Cell viability curves of human glioma SF763 cells treated with NBGNU, ACNU and ACNU plus O⁶-BG (20 μM) under hypoxia and normoxia. Typical SRM chromatograms of dGuo-dCyd and ¹⁵N₃-dGuo-dCyd as an internal standard in DNA from (B) control SF763 cells and from (C) cells treated with NBGNU under hypoxia. The determined levels of dGuo-dCyd crosslink in cells treated with NBGNU, ACNU and ACNU plus O⁶-BG (20 μM) under hypoxic and normoxic conditions. (D) Drug concentration of 0.2 mM in SF763 cells; (E) drug concentration of 0.6 mM in SF763 cells; (F) drug concentration of 0.2 mM in SF126 cells; (G) drug concentration of 0.6 mM in SF126 cells. Data are presented as the mean ± SD (n = 3).

*p < 0.05; **p < 0.01.

O⁶-BG: O⁶-benzylguanine; SRM: Selecting reaction monitoring.

O⁶-BG derivatives released from NBGNU and therefore enhanced the induction of dGuo-dCyd crosslinks, which might cause NBGNU to inhibit AGT-expressing cells in the hypoxic area of solid tumors in a targeted manner. To confirm the inhibition of AGT activity by the reduction product released from NBGNU under hypoxia, the AGT activity in SF126 and SF763 cells was measured using HPLC–ESI–MS/MS analysis reported in our previous study [43]. The results indicated that no AGT activity was observed in the AGT-proficient SF763 cells under hypoxia. This provided direct evidence that the O⁶-BG derivatives, which were released from NBGNU under hypoxia, possess high AGT inhibitory activity in SF763 cells. For the NBGNU-treated SF763 cells under normoxia, a low level of AGT activity (close to the detection of limit of the method and approximately <10 fmol/mg protein extract) was observed, which might contribute to the relatively lower crosslinking levels than those under hypoxia. In all SF126 samples, no substantial level of AGT activity was detectable using the supposed method.

Interaction with human AGT by docking simulation

To clarify the discrimination of AGT inhibition under hypoxic and normoxic conditions, the interaction between the substrates yielded from NBGNU and the active pocket was explored by molecular docking. All possible substrates (compounds 1–6 in Figure 2), including NBGNU and its decomposition or reduction products yielded under normoxic or hypoxic conditions, were docked with AGT. The molecular structures of all AGT-substrate complexes obtained from docking are shown in Figure 6. Among the complexes D–F of AGT with the reduction products 4–6, respectively, the ligands completely entered into the active pocket and four H-bonds formed between the ligands and four residues (Ser159, Asn157, Cys145 and Val148) with the Cys145 residue locating near to the O⁶-benzyl group. However, in complexes A–C, consisting of AGT interacting with 1 (NBGNU) and its decomposition products 2 and 3, respectively, generated under normoxic conditions, there are only one or two hydrogen bonds formed between the ligands and a single residue (Tyr114, Arg135 or Ser159). Moreover, the docked poses of complexes A–C provided a following definitive explanation: the ligands did not substantially enter into the active pocket of the protein and adopted inappropriate orientations in which the O⁶-benzyl group remained

Figure 5. Cell viability and DNA ICLs in human glioma cells treated with NBGNU under normoxia and hypoxia (cont.). (A) Cell viability curves of human glioma SF763 cells treated with NBGNU, ACNU and ACNU plus O⁶-BG (20 μ M) under hypoxia and normoxia. Typical SRM chromatograms of dGuo-dCyd and ¹⁵N₃-dGuo-dCyd as an internal standard in DNA from (B) control SF763 cells and from (C) cells treated with NBGNU under hypoxia. The determined levels of dGuo-dCyd crosslink in cells treated with NBGNU, ACNU and ACNU plus O⁶-BG (20 μ M) under hypoxic and normoxic conditions. (D) Drug concentration of 0.2 mM in SF763 cells; (E) drug concentration of 0.6 mM in SF763 cells; (F) drug concentration of 0.2 mM in SF126 cells; (G) drug concentration of 0.6 mM in SF126 cells. Data are presented as the mean $\pm$ SD (n = 3).

*p < 0.05; **p < 0.01.

O⁶-BG: O⁶-benzylguanine; SRM: Selecting reaction monitoring.

far away from the Cys145 residue, which disabled the transferring of the O⁶-benzyl group to the Cys145 residue and resulted in the failure of AGT inhibition.

Discussion

As reported in previous studies, the 2-amino group on guanine is necessary for effective inhibition of AGT activity because the ligand is combined with the enzyme by a crucial hydrogen bond between the 2-amino group and the active pocket [9,20]. Based on this conclusion, the tripartite prodrug NBGNU was designed such that the 4-nitrobenzyloxycarbonyl group masking the 2-amino group is removed via nitroreduction under hypoxia, resulting in a hypoxia-specific inhibition of AGT activity and thus sensitizing cancer cells to the CENU pharmacophore. In contrast, under the aerobic conditions in normal tissue, the nitro group in the 4-nitrobenzyloxycarbonyl group will not undergo reduction, leading to the retention of AGT activity and the protection of normal cells from O⁶-guanine alkylation.

Figure 6. Structures of the complexes of AGT (PDB code: 1QNT) with the reduction or decomposition products obtained from docking with the best-scoring conformation predicted by Gold Score. The structures of complexes A–F correspond to the combination of AGT with compounds 1–6, respectively. The hydrogen bonding interactions between the ligands and the amino acid residues in the active pocket of the protein are shown as yellow dash lines in each complex.

To confirm the hypoxia-activated effect of NBGNU, enzymatically generated oxygen-deficient systems in PBS and in cell suspension were used to simulate the hypoxic tumor microenvironment. The amount of the reduction product was determined and compared under normoxic and hypoxic conditions to confirm the selective hypoxic activation of NBGNU. The reduction product AEBG was observed in all hypoxic samples but not in any normoxic samples in PBS (Figure 4A & B). These results indicated that NBGNU selectively underwent enzymatic reduction to release an AGT inhibitor under hypoxia but not under normoxia. As shown in Figure 4E, the highest yield of AEBG in PBS was 860 nM, which was obtained with 10 mM xanthine and 1.6 U/ml xanthine oxidase after 8 h of incubation and corresponded to 9% reduction of the parent compound. Zhu *et al.* [37] reported several *O*⁶-BG

derivatives containing 4-nitrobenzyloxycarbonyl group as AGT inhibitors. They observed an approximately 14% yield of the reduction product (O^6 -BG) in a hypoxic system generated by xanthine/xanthine oxidase, which was slightly higher than the yield of AEBG (9%) observed in this study owing to the CENU moiety decomposing to other products than AEBG. In contrast to the PBS samples, AEBG was also observed in normoxic cell suspensions (Figure 4C), but the yield was as low as approximately 85 nM, accounting for only 0.09% reduction of the corresponding parent compound. Moreover, in cell suspension (Figure 4F), the amount of AEBG under hypoxia mildly increased with the incubation time and approximately 11.3% of the parent compound was reduced at 8 h (accounting for 11340 nM AEBG), whereas the yield of AEBG under normoxia remained almost constant after 2 h, which also evidenced that the reduction mechanism under normoxia might be different from that under hypoxia. Previous studies by Shyam *et al.* [33,52] revealed that a glutathione/glutathione transferase-mediated thiolysis occurred by attacking the methylene on the nitrobenzyl group, which contributed to an O_2 concentration-independent activation mechanism. This provided a plausible explanation for the small normoxic cytotoxicity of NBGNU. In addition to thiolysis, reduction of the 4-nitrobenzyl moiety by two electron reductases such as NQO1 could also contribute to normoxic cytotoxicity.

As described in a number of studies, CENUs undergo spontaneous decomposition to generate diazonium ions and carbon cations to act as DNA alkylating agents. Therefore, the decomposition kinetics of NBGNU was explored in this study. As shown in Figure 3, the half-life of NBGNU in cell suspension was approximately 18 min, which was longer than that of ACNU in plasma (12 min) [51]. In our previous studies, the CENU-induced dGuo-dCyd crosslink in calf thymus DNA started to undergo a rapid accumulation after an 'induction period' of approximately 2 h [40,53]. This suggested that the covalent bonding of O^6 -ClEtG with the complementary cytosine residue was the rate-limiting step for crosslink formation; moreover, O^6 -ClEtG, which was the precursor of the dGuo-dCyd crosslinks and a substrate of AGT, was mainly yielded within the first 2 h of the drug treatment. As observed in this study, the hypoxic activation of NBGNU was almost completed within 1 h followed by slight increase of the AEBG concentration. Consequently, there should be enough time for the depletion of AGT activity by the O^6 -BG derivatives released from hypoxic activation before O^6 -ClEtG reacts with cytosine, facilitating the formation of dGuo-dCyd crosslinks. This mechanism ensures the effective action of the CENU pharmacophore after AGT activity was completely depleted. Similarly, a fast reduction of the nitrobenzyl group under hypoxia was also observed in the studies of Seow *et al.* [34], who reported that the reduction of the 4-nitrobenzyloxycarbonyl moiety to amino could be rapidly accomplished in a hypoxic Zn/EDTA solution with a half-life of approximately 30 s.

High AGT-expressing SF763 cells were employed to examine the anticancer activity of NBGNU. The activity of NBGNU under hypoxic conditions ($IC_{50} = 126 \mu M$) was significantly higher than that of NBGNU under normoxic conditions ($IC_{50} = 580 \mu M$) and ACNU under both hypoxic and normoxic conditions ($IC_{50} > 1000 \mu M$). The results indicated that NBGNU selectively underwent reduction under hypoxic condition, leading to unmasking of the 2-amino group of guanine, release of O^6 -BG analogs and effective inhibition of AGT. As expected, SF763 cells showed increased sensitivity toward NBGNU under hypoxia. Moreover, the observed IC_{50} value of NBGNU was >8-times lower than that of ACNU plus O^6 -BG ($IC_{50} > 1000 \mu M$). This could be attributed to the theoretically equimolar amounts of AGT inhibitor and alkylating agent released from NBGNU, whereas the concentration of O^6 -BG in the combination treatment was fixed at $20 \mu M$. Notably, the inhibitory activity of NBGNU under normoxia was also obviously higher than that of ACNU and was similar to that of the combination treatment. The NBGNU prodrug was expected to exert similar activity to ACNU under normoxia because of the 2-amino-masked O^6 -BG derivative, leading to the loss of AGT inhibition. However, as described above, the 4-nitrobenzyloxycarbonyl group could be slightly reduced under normoxic conditions and induce AGT inhibition to a minor extent, contributing to the observed cytotoxicity of NBGNU under normoxia.

In accordance with the results of the hypoxic activation and cell viability assays, the ability of NBGNU to induce dGuo-dCyd crosslink was higher in the hypoxic group than in any of the other groups. The high crosslinking levels, high yield of the reduction product and low IC_{50} value of NBGNU observed under hypoxia all demonstrated that the prodrug synthesized in this study achieved the desired objectives; it simultaneously achieved hypoxic selectivity and elimination of chemoresistance to CENUs. The levels of NBGNU-induced dGuo-dCyd crosslink under hypoxia were approximately four-times higher ($p < 0.01$) than those under normoxia, which was roughly in accordance with the difference in the IC_{50} values of NBGNU under hypoxia ($126 \mu M$) and normoxia ($580 \mu M$). This suggested that the dGuo-dCyd crosslinking level could be utilized as an indicator of the chemotherapeutic efficacy of the combination of nitrosoureas, as predicted in our previous studies on traditional CENUs [40,53]. Moreover, in SF763 cells under

normoxia, the levels of NBGNU-induced crosslinks were higher than those by ACNU, which was consistent with the lower IC₅₀ value of NBGNU than ACNU under normoxia in the cytotoxicity assay but was inconsistent with our expectations. As mentioned above, this might be attributed to the glutathione/glutathione transferase-mediated oxygen-independent activation mechanism. Another concern of the combi-nitrosourea prodrug is indispensable that dose limits may be imposed by the release of the CENU moiety in normal tissues. It is possible that the dose can be independently adjusted to ensure maximal target engagement of AGT, which may be achieved with two separate agents consisting of a tumor targeting CENU plus a hypoxia-activated AGT inhibitor.

Conclusion

A tripartite combi-nitrosourea prodrug, NBGNU, which combines a CENU pharmacophore with an *O*⁶-BG derivative masked by a 4-nitrobenzyl-oxycarbonyl group on its 2-amino group, was developed in this study, and its anticancer potency was evaluated by *in vitro* experiments. The prodrug exhibits desirable antitumor efficacy and hypoxic selectivity owing to the introduction of the AGT inhibitor and the hypoxia-activated pharmacophores into the side chain of the CENU moiety. Nevertheless, the hypoxic selectivity and chemotherapeutic efficacy of the prodrug still need to be improved by eliminating normoxia reduction and enhancing water solubility. To achieve these goals, several new combi-nitrosoureas with modified substructures are under development in our lab by introducing other reducible moieties with higher hypoxic selectivity and endowing the molecules with appropriate lipo/hydrosolubility.

Future perspective

Although CENUs exhibit efficacy against leukemia, cerebroma and various solid tumors, they are limited to further clinical application because of drug resistance and high side effect. To improve the chemotherapeutic effect of this type of agents, great efforts have been devoted to develop novel compounds by introducing various moieties to optimize the molecular structures of CENUs, including synthesizing new combi-molecules with dual or tripartite properties to facilitate the high efficacy and tumor targeting of this chemotherapy. However, failures are inevitable in the development of novel combi-nitrosoureas and new challenges are emerging in the discovery of such therapies. Therefore, in the future, the following aspects should be considered in the design and synthesis of hypoxia-activated combi-nitrosourea prodrug. First, high selectivity toward hypoxia of the prodrug needs to be focused to improve tumor targeting by eliminating normoxia reduction. Second, as bone marrow having hypoxic/low oxia compartments, profound and prolonged myelosuppression have to be cautious and therefore *in vivo* evidence are need to demonstrated the superiority of hypoxia-activated combi-nitrosourea prodrug over conventional CENUs. Third, the novel combi-nitrosoureas need to possess desirable pharmacodynamics and pharmacokinetics properties to satisfy the requirements in further clinical application.

Summary points

- A hypoxia-activated combi-nitrosourea prodrug, N-(2-chloroethyl)-N'-2-(2-(4-nitrobenzylcarbamate)-*O*⁶-benzyl-9-guanine)ethyl-N-nitrosourea (NBGNU), was synthesized as a tripartite molecule consisting of a chloroethylnitrosourea pharmacophore, an *O*⁶-benzylguanine (*O*⁶-BG) analog moiety and a hypoxia-activatable 4-nitrobenzylcarbamate group.
- The target compound undergoes hypoxia activation in an enzymatic reduction mixture containing glucose/glucose oxidase, catalase and xanthine/xanthine oxidase.
- Significantly higher chemosensitivity was observed in human glioma SF763 cells (high *O*⁶-alkylguanine-DNA alkyltransferase-expression) treated by NBGNU under hypoxia compared with those under normoxia and those treated by 1-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea (ACNU) and ACNU + *O*⁶-BG.
- The levels of DNA interstrand crosslink induced by NBGNU under hypoxia are remarkably higher than those under normoxia and those induced by ACNU and ACNU + *O*⁶-BG.
- Molecular docking suggested that the *O*⁶-BG analogs released from NBGNU under hypoxia could inhibit *O*⁶-alkylguanine-DNA alkyltransferase activity by entering the active pocket of the protein.
- NBGNU exhibits expected hypoxia selectivity suggesting potential tumor hypoxia targeting in further *in vivo* studies.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science/doi/suppl/10.4155/fmc-2018-0511

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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References

Papers of special note have been highlighted as: ● of interest; ●● of considerable interest

1. Larsson AK, Shokeer A, Mannervik B. Molecular evolution of theta-class glutathione transferase for enhanced activity with the anticancer drug 1,3-bis-(2-chloroethyl)-1-nitrosourea and other alkylating agents. *Arch. Biochem. Biophys.* 497(1–2), 28–34 (2010).
2. Das D, Preet R, Mohapatra P, Satapathy SR, Kundu CN. 1,3-Bis(2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells. *World J. Gastroenterol.* 19(42), 7374–7388 (2013).
3. Chamberlain MC. Anticancer therapies and CNS relapse: overcoming blood-brain and blood-cerebrospinal fluid barrier impermeability. *Exp. Rev. Neurother.* 10(4), 547–561 (2010).
4. Chen FX, Bodell WJ, Liang GN, Gold B. Reaction of N-(2-chloroethyl)-N-nitrosoureas with DNA: effect of buffers on DNA adduction, cross-linking, and cytotoxicity. *Chem. Res. Toxicol.* 9(1), 208–214 (1996).
5. Daniels DS, Woo TT, Luu KX *et al.* DNA binding and nucleotide flipping by the human DNA repair protein AGT. *Nat. Struct. Mol. Biol.* 11(8), 714–720 (2004).
- **A critical investigation on the crystal structure of AGT and the mechanism of action of AGT-mediated repair of O⁶-alkylguanines.**
6. Tubbs JL, Pegg AE, Tainer JA. DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O⁶-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. *DNA Repair* 6(8), 1100–1115 (2007).
7. Pegg AE. Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools. *Chem. Res. Toxicol.* 24(5), 618–639 (2011).
- **An excellent review that describes the role of O⁶-Alkylguanine-DNA alkyltransferase (AGT) in DNA repair, DNA damage, resistance to chemotherapy and related research tools.**
8. Esteller M, Hamilton SR, Burger PC. Inactivation of the DNA repair gene O⁶-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.* 59(4), 793–797 (1999).
9. Sun GH, Fan TJ, Zhang N, Ren T, Zhao LJ, Zhong RG. Identification of the structural features of guanine derivatives as MGMT inhibitors using 3D-QSAR modeling combined with molecular docking. *Molecules* 21(7), 823 (2016).
- **An interesting investigation that provides an insight into the structure–activity relationship of guanine derivatives as AGT inhibitors.**
10. Dolan ME, Pegg AE, Moschel RC, Grindey GB. Effect of O⁶-benzylguanine analogs on the sensitivity of human colon tumor xenografts to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.* 46(2), 285–290 (1993).
11. Pegg AE, Boosalis M, Samson L *et al.* Mechanism of inactivation of human O⁶-alkylguanine-DNA alkyltransferase by O⁶-benzylguanine. *Biochem.* 32(45), 11998–12006 (1993).
12. Dolan ME, Pegg AE. O⁶-benzylguanine and its role in chemotherapy. *Clin. Cancer Res.* 3(6), 837–847 (1997).

13. Bacolod MD, Johnson SP, Pegg AE *et al.* Brain tumor cell lines resistant to O⁶-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy have O⁶-alkylguanine-DNA alkyltransferase mutations. *Mol. Cancer Ther.* 3(9), 1127–1135 (2004).
14. Batts ED, Maisel C, Kane D *et al.* O⁶-Benzylguanine and BCNU in multiple myeloma: a phase II trial. *Cancer Chemother. Pharmacol.* 60(3), 415–421 (2007).
15. Warren KE, Gururangan S, Geyer JR *et al.* A Phase II study of O⁶-benzylguanine and temozolomide in pediatric patients with recurrent or progressive high grade gliomas and brainstem gliomas: a pediatric brain tumor consortium study. *J. Neurooncol.* 106(3), 643–649 (2012).
16. Sun GH, Zhao LJ, Zhong RG. The induction and repair of DNA interstrand crosslinks and implications in cancer chemotherapy. *Anticancer Agents Med. Chem.* 16(2), 221–246 (2016).
17. McElhinney RS, Donnelly DJ, McCormick JE *et al.* Inactivation of O⁶-alkylguanine-DNA alkyltransferase. 1. novel O⁶-(hetaryl-methyl)-guanines having basic rings in the side chain. *J. Med. Chem.* 41(26), 5265–5271 (1998).
18. Ranson M, Middleton MR, Bridgewater J *et al.* Lomeguatrib, a potent inhibitor of O⁶-alkylguanine-DNA-alkyltransferase: Phase I safety, pharmacodynamic, and pharmacokinetic trial and evaluation in combination with temozolomide in patients with advanced solid tumors. *Clin. Cancer Res.* 12(5), 1577–1584 (2006).
19. Pauly GT, Loktionova NA, Fang Q, Vankayala SL, Guida WC, Pegg AE. Substitution of aminomethyl at the metaposition enhances the inactivation of O⁶-alkylguanine-DNA alkyltransferase by O⁶-benzylguanine. *J. Med. Chem.* 51(22), 7144–7153 (2008).
20. Chae MY, Swenn K, Kanugula S, Dolan ME, Pegg AE, Moschel RC. 8-Substituted O⁶-benzylguanine, substituted 6(4)-(benzyloxy)pyrimidine, and related derivatives as inactivators of human O⁶-alkylguanine-DNA alkyltransferase. *J. Med. Chem.* 38(2), 359–365 (1995).
21. Terashima I, Kohda K. Inhibition of human O⁶-alkylguanine-DNA alkyltransferase and potentiation of the cytotoxicity of chloroethylnitrosourea by 4(6)-(benzyloxy)-2,6(4)-diamino-5-(nitro or nitroso) pyrimidine derivatives and analogues. *J. Med. Chem.* 41(4), 503–508 (1998).
22. Fortin S, Berube G. Advances in the development of hybrid anticancer drugs. *Expert. Opin. Drug Discov.* 8(8), 1029–1047 (2013).
23. Wanner MJ, Koch M, Koomen GJ. Synthesis and antitumor activity of methyltriazene prodrugs simultaneously releasing DNA-methylating agents and the anti-resistance drug O⁶-benzylguanine. *J. Med. Chem.* 47(27), 6875–6883 (2004).
24. Brahimi F, Rachid Z, Qiu QY *et al.* Multiple mechanisms of action of ZR2002 in human breast cancer cells: a novel combi-molecule designed to block signaling mediated by the ERB family of oncogenes and to damage genomic DNA. *Int. J. Cancer* 112(3), 484–491 (2004).
25. Rachid Z, Brahimi F, Domarkas J, Jean-Claude BJ. Synthesis of half-mustard combi-molecules with fluorescence properties: correlation with EGFR status. *Bioorg. Med. Chem. Lett.* 15(4), 1135–1138 (2005).
26. Qiu QY, Domarkas J, Banerjee R *et al.* The combi-targeting concept: *in vitro* and *in vivo* fragmentation of a stable combi-nitrosourea engineered to interact with the epidermal growth factor receptor while remaining DNA reactive. *Clin. Cancer Res.* 13(1), 331–340 (2007).
27. Qiu QY, Dudouit F, Banerjee R, McNamee JP, Jean-Claude BJ. Inhibition of cell signaling by the combi-nitrosourea FD137 in the androgen independent DU145 prostate cancer cell line. *Prostate* 59(1), 13–21 (2004).
28. Domarkas J, Dudouit F, Williams C *et al.* The combi-targeting concept: synthesis of stable nitrosoureas designed to inhibit the epidermal growth factor receptor (EGFR). *J. Med. Chem.* 49(12), 3544–3552 (2006).
29. Sun GH, Zhang N, Zhao LJ, Fan TJ, Zhang SF, Zhong RG. Synthesis and antitumor activity evaluation of a novel combi-nitrosourea prodrug: designed to release a DNA cross-linking agent and an inhibitor of O⁶-alkylguanine-DNA alkyltransferase. *Bioorg. Med. Chem.* 24(9), 2097–2107 (2016).
- **An important investigation on the development of a novel combi-nitrosourea prodrug, which releases DNA cross-linking agent and AGT inhibitor to enhance chemosensitivity.**
30. Plavetic ND, Baric M, Bradic L, Kulic A, Plestina S. Hypoxia in solid tumors: biological responses to hypoxia and implications on therapy and prognosis. *Period Biol.* 116(4), 361–364 (2014).
31. Wigerup C, Pahlman S, Bexell D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacol. Ther.* 164(1), 152–169 (2016).
32. Shyam K, Penketh PG, Baumann RP *et al.* Antitumor sulfonylhydrazines: design, structure–activity relationships, resistance mechanisms, and strategies for improving therapeutic utility. *J. Med. Chem.* 58(9), 3639–3671 (2015).
33. Shyam K, Penketh PG, Shapiro M *et al.* Hypoxia-selective nitrobenzyloxycarbonyl derivatives of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. *J. Med. Chem.* 42(5), 941–946 (1999).
- **A typical example of hypoxia-activated AGT inhibitors with tumor targeting ability.**
34. Seow HA, Penketh PG, Shyam K, Rockwell S, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine: an anticancer agent targeting hypoxic cells. *Proc. Natl Acad. Sci. USA* 102(26), 9282–9287 (2005).

35. Baumann RP, Penketh PG, Ishiguro K, Shyam K, Zhu YL, Sartorelli AC. Reductive activation of the prodrug 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119) selectively occurs in oxygen-deficient cells and overcomes O⁶-alkylguanine-DNA alkyltransferase mediated KS119 tumor cell resistance. *Biochem. Pharmacol.* 79(11), 1553–1561 (2010).
36. Baumann RP, Ishiguro K, Penketh PG, Shyam K, Zhu R, Sartorelli AC. KS900: a hypoxia-directed, reductively activated methylating antitumor prodrug that selectively ablates O⁶-alkylguanine-DNA alkyltransferase in neoplastic cells. *Biochem. Pharmacol.* 81(10), 1201–1210 (2011).
37. Zhu R, Liu MC, Luo MZ *et al.* 4-Nitrobenzyloxycarbonyl derivatives of O⁶-benzylguanine as hypoxia-activated prodrug inhibitors of O⁶-alkylguanine-DNA alkyltransferase (AGT), which produces resistance to agents targeting the O-6 position of DNA guanine. *J. Med. Chem.* 54(21), 7720–7728 (2011).
- **A typical example of hypoxia-activated AGT inhibitors with tumor targeting ability.**
38. Zhu R, Seow HA, Baumann RP *et al.* Design of a hypoxia-activated prodrug inhibitor of O⁶-alkylguanine-DNA alkyltransferase. *Bioorg. Med. Chem. Lett.* 22(19), 6242–6247 (2012).
39. Penketh PG, Shyam K, Baumann RP *et al.* A strategy for selective O⁶-alkylguanine-DNA alkyltransferase depletion under hypoxic conditions. *Chem. Biol. Drug Des.* 80(2), 279–290 (2012).
40. Bai BQ, Zhao LJ, Zhong RG. Quantification of meCCNU-induced dGuo-dCyd crosslinks in oligonucleotide duplexes by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Sp.* 25(14), 2027–2034 (2011).
41. Boddell WJ, Pongracz K. Chemical synthesis and detection of the cross-link 1-[N³-(2'-deoxycytidyl)]-2-[N¹-(2'-deoxyguanosinyl)]ethane in DNA reacted with 1-(2-chloroethyl)-1-nitrosourea. *Chem. Res. Toxicol.* 6(4), 434–438 (1993).
42. Baumann RP, Penketh PG, Seow HA, Shyam K, Sartorelli AC. Generation of oxygen deficiency in cell culture using a two-enzyme system to evaluate agents targeting hypoxic tumor cells. *Radiat. Res.* 170(5), 651–660 (2008).
43. Sun GH, Zhao LJ, Fan TJ, Ren T, Zhong RG. Measurement of O-6-alkylguanine-DNA alkyltransferase activity in tumour cells using stable isotope dilution HPLC–ESI–MS/MS. *J. Chromatogr. B* 1033(15), 138–146 (2016).
44. Bobola MS, Blank A, Berger MS, Silber JR. Contribution of O⁶-methylguanine-DNA methyltransferase to monofunctional alkylating-agent resistance in human brain tumor-derived cell lines. *Mol. Carcinogen.* 13(2), 70–80 (1995).
45. Wang YM, Ren T, Lai XX *et al.* Synthesis and antitumor activity evaluation of a novel combi-nitrosourea prodrug: BGCNU. *ACS Med. Chem. Lett.* 8(2), 174–178 (2017).
- **An important investigation on the design, synthesis and anticancer activity evaluation of novel combi-nitrosourea prodrug.**
46. Li LL, Zhao LJ, Zhong RG. Quantification of DNA interstrand crosslinks induced by ACNU in NIH/3T3 and L1210 cells using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Sp.* 28(5), 439–447 (2014).
47. Sun GH, Zhao LJ, Fan TJ, Li SS, Zhong RG. Investigations on the effect of O⁶-benzylguanine on the formation of dGuo-dCyd interstrand cross-links induced by chloroethylnitrosoureas in human glioma cells using stable isotope dilution high-performance liquid chromatography electrospray ionization tandem mass spectrometry. *Chem. Res. Toxicol.* 27(7), 1253–1262 (2014).
48. Zhao LJ, Balbo S, Wang MY *et al.* Quantitation of pyridyloxobutyl-DNA adducts in tissues of rats treated chronically with (R)- or (S)-N'-nitrosomornicotine (NNN) in a carcinogenicity study. *Chem. Res. Toxicol.* 26(10), 1526–1535 (2013).
49. Frisch MJ, Trucks GW, Schlegel HB *et al.* Gaussian 09, Revision A.01, Gaussian Inc., Wallingford, CT, USA (2009).
50. Wiencke JK, Wiemels J. Genotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Mutat. Res.* 339(2), 91–119 (1995).
51. Tanaka M, Nishigaki T, Nakajima E, Totsuka S, Nakamura K. Distribution, excretion, and metabolism of 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU) in rats and mice after iv administration. *Cancer Treat. Rep.* 64(4–5), 575–580 (1980).
52. Shyam K, Penketh PG, Loomis RH, Sartorelli AC. Thiolysable prodrugs of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine with antineoplastic activity. *Eur. J. Med. Chem.* 33(7–8), 609–615 (1998).
53. Zhao LJ, Li LL, Xu J, Zhong RG. Comparative investigation of the DNA inter-strand crosslinks induced by ACNU, BCNU, CCNU and FTMS using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Int. J. Mass Spectrom.* 368(15), 30–36 (2014).